PATENT
Docket No.: **201487/1030**

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants	:	Karube et al.	Examiner: Bronwen M. Loeb
Serial No.	:	CPA of 09/623,970	
Cnfrm. No.	:	1866	Art Unit: 1636
Filed	:	March 12, 1999	
For	:	SITE-SPECIFIC CELL PERFORATION TECHNIQUE	

DECLARATION OF TAKASHI SAITOH UNDER 37 CFR § 1.132

Mail Stop
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

I, TAKASHI SAITOH, pursuant to 37 C.F.R. § 1.132, declare:

1. I am a co-inventor of the above-identified patent application.

2. This declaration is submitted to demonstrate that site-specific regulated membrane penetration of my above application can be performed with other combinations of membrane-disrupting reagents and stimuli besides the photosensitizer-light combination already exemplified in the specification of my patent application. The experiment described here shows the method of sonodynamic therapy applied to a membrane penetration system where a sample cell is contacted with a capillary containing a membrane-disrupting reagent and an injection marker and stimulated by sonication with a piezoelectric immersion transducer.

3. PC12 rat pheochromocytoma cells were obtained from the RIKEN cell bank for the sample cell line. The cells were cultured as described in Saito et al., "Light Dose and Time Dependency of Photodynamic Cell Membrane Damage," Photochem. Photobiol. 68:745-748 (1998), except that serum-free Neurobasal™ supplemented with B27 (Invitrogen, Carlsbad, CA) and containing 7.35 mg/l L-glutamic acid, 2 mM L-glutamine was used as the

culture medium. Frozen-thawed cells were cultured at the density of 6 cells/μl, with 3000 μl of the medium in a collagen type I-coated culture dish (ASAHI TECHNO GLASS Corp., Japan) and were used after one week.

4. The injection composition consisted of: 140 mM KCl, 8 mM NaCl, 10 mM HEPES (pH 7.3), 0.5 mM $MgCl_2$, 2 mM Lucifer Yellow CH (Molecular Probes, Inc., Eugene, OR) as the fluorescecnt labeling dye, and 20 μM Merocyanine 540 (Aldrich Chem. Co., Milwaukee, WI) as the sonosensitizer. The active frequency of Merocyanine 540 was selected from Tachibana et al., "Introduction of Cell-Membrane Porosity by Ultrasound," Lancet, 353:1409 (1999). For the control experiment, the injection composition was prepared without the sonosensitizer.

5. For the stimulation, a piezoelectric immersion transducer (KRAUTKRÄMER 2.25 MHz Ultrasound Xducer P/N 113-142-300 0.5" AED Alpha Series, ISS style non-Focused, Product Code 113-142-300, Agfa-Gevaert N.V., Belgium) was used to perform sonication. An operational signal amplifier (PA85 (APEX Microtechnology Corp., Tucson, U.S.A.)), a function generator (33120A (Hewlett-Packard, Palo Alto, U.S.A.) (driving frequency: 255 kHz; amplitude: 900 mV; rectangular wave)), and a DC power supply (TMP-5500 (TAITEC Co. Ltd., Japan) (+100V) and Model 500/1000 (Bio-Rad Laboratories, Inc., Hercules, U.S.A.) (-100 V)) were used in conjunction with the transducer. The final driving voltage amplitude of the piezoelectric oscillator was 180 V peak to peak, and the calculated input power was 1.84 W/cm^2. The observed PZT driving waveform was dulled as trapezoid wave and its slew rate was 120 V/microsec. For the stimulating acoustic power evaluation, a hydrophone (TNU100A (NTRsystems, Inc. Seattle, U.S.A.)) with a 30 dB preamp, driven by DC power supply PAN35-10A (KIKUSUI Electronics Corp., Japan) at 15.0 V and calibrated by the output value at 0.4 MHz) was used.

6. An inverted fluorescence microscope IMT-2 (OLYMPUS Optics Co., Ltd. Japan) was used. The excitation light from a 100 W-mercury lamp unit (NIKON Corp., Japan) was transmitted through a blue excitation filter set (U-MWB, combining a λ = 450 to 480-nm band pass filter for excitation, a λ_c = 500-nm Dichroic mirror, and a λ_c = 515-nm sharp cut filter for emission (OLYMPUS Optics Co., Ltd. Japan)) for Lucifer Yellow CH excitation. A rack and pinion driven manipulator for positioning the hydrophone and air-CO_2 mixed gas blowing tube and a hydraulic micromanipulator, MHW-3 (NARISHIGE Co. Ltd., Japan) for positioning an injection capillary (Femtotip I (Eppendorf AG, Germany)) and the ultrasonic

oscillator, were used. Material was fed to the injection capillary with a microsyringe (IM-6 (NARISHIGE Co. Ltd., Japan)).

7. PC12 rat pheochromocytoma cells, prepared as described above, were placed in a culture dish operatively positioned with respect to the membrane penetration system described above and shown in attached Figure 1. The tips of the injection capillary and the hydrophone were placed against a cell. As a result of sonication with the oscillator, the membrane of the cell was penetrated and reagents were injected into the cell by the microsyringe via the injection capillary. The injection process was viewed through the microscope with flow leakage of the marker being observed for 120 sec in a static condition and for 120 sec when the sonication was performed. The leak flow of the injection was accelerated by the sonication. This process was stopped when the cell was stained. To keep the pH of the medium below 7.6 during operation, 95% air and 5% CO_2 mixed gas was blown on the surface of the medium at a pressure of 0.08 MPa.

8. The results of the above experiments are depicted in attached Table 1 and Figures 2 and 3 and can be classified into the following four groups:

Group A: Injection occurred before sonic stimulation.

Group B: Injection occurred immediately after the initiation of sonic stimulation.

Group C: Injection occurred after the initiation of sonic stimulation with time delay.

Group D: Injection failed.

In the control experiments where sonosensitizers were not applied, 1 cell out of total 24 cells showed time delayed penetration after sonication (Cell No. 13 of Figure 2 and Table 1). This penetration was likely triggered by mechanical shock during the sonication, since the sonosensitizing reaction should not occur in the absence of sonosensitizer. Thus, in the control experiments, there was a total of 12 cells (Groups A, B, and C) that could be considered to be penetrated due to mechanical shock. Since there was 1 cell that showed time delayed penetration after sonication out of the 13 cells which remained unpenetrated after the initiation of sonic stimulation (Groups C and D), the probability of this unpredictable penetration triggered by mechanical shock was about 8%. On the other hand, in experiments where sonosensitizers were applied, 8 cells out of 24 cells displayed time

delayed penetration after sonication (Cell Nos. 5-11 and 23 of Figure 3 and Table 1). Since there were 12 cells which remained unpenetrated after the initiation of sonic stimulation (Groups C and D), about 0 or 1 cell (8% of 12 cells) was likely penetrated due to mechanical shock during the sonication. Therefore, it can be estimated that 8 or 7 cells, which are classified as Group C, were penetrated by the sonosensitizing reaction, while there was a total of 12-13 cells (Groups A, B, and C) that could be considered to be penetrated due to mechanical shock. In sum, the injection success ratio was improved from 0 cell to 7 or 8 cells by applying a sonosensitizer. Although the injection success ratio triggered by mechanical shock showed little difference with and without sonosensitizer, 50-54% (12-13 /24) and 50% (12/24), respectively, the increase in injection success ratio by using a sonosensitizer, 29-33% (7-8 /24), was significant. These results show that site-specific regulated membrane penetration can be performed with a combination of a sonosensitizer and sonication.

9. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Date: June 27, 2003

Takashi Saitoh

Takashi Saitoh

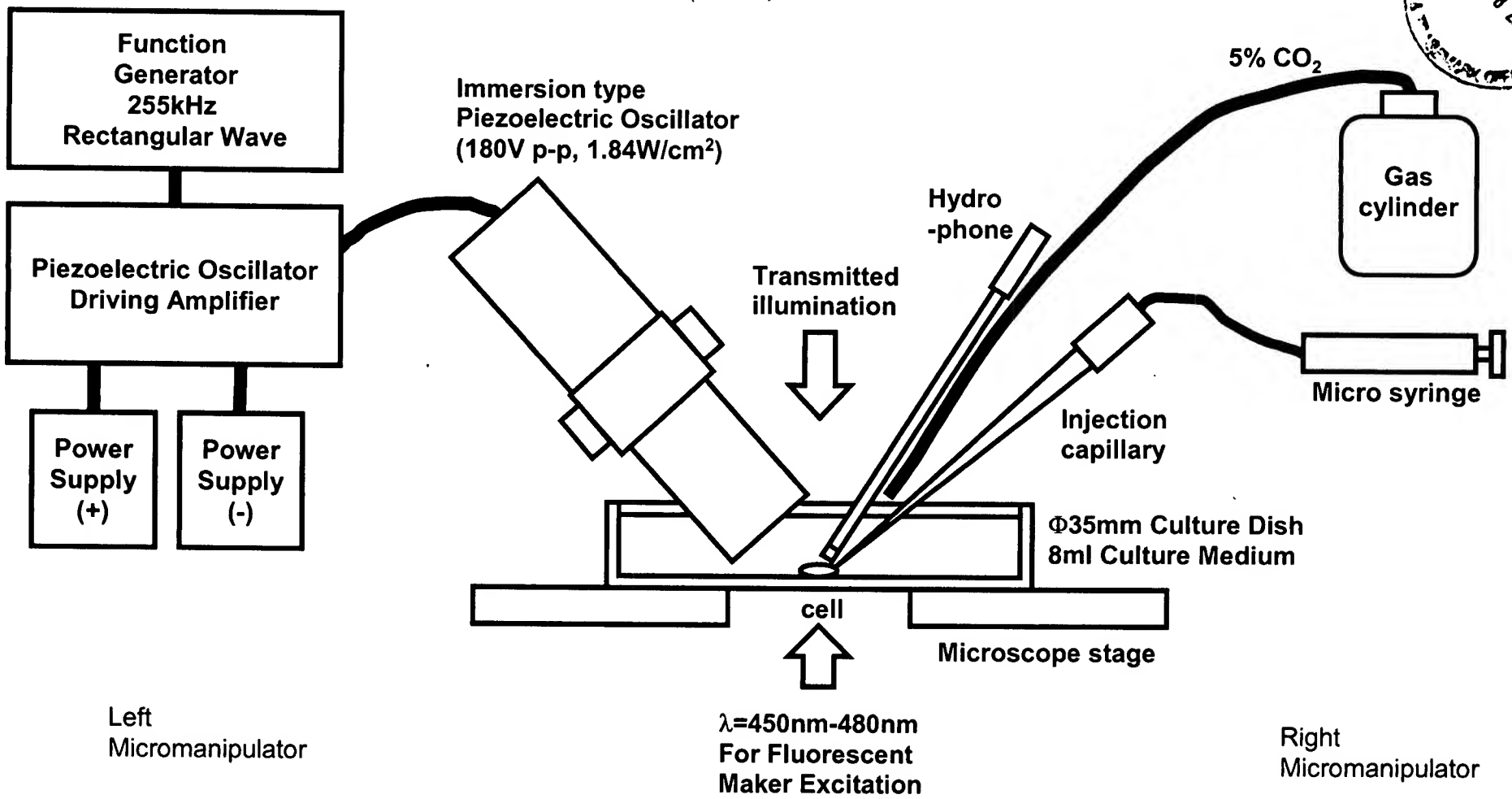

Fig. 1 Membrane penetration system performed by Sonication with piezoelectric transducer as an ultrasonic oscillator.
Based on an inverted fluorescence microscope IMT-2 (OLYMPUS Optics Co., Ltd.)

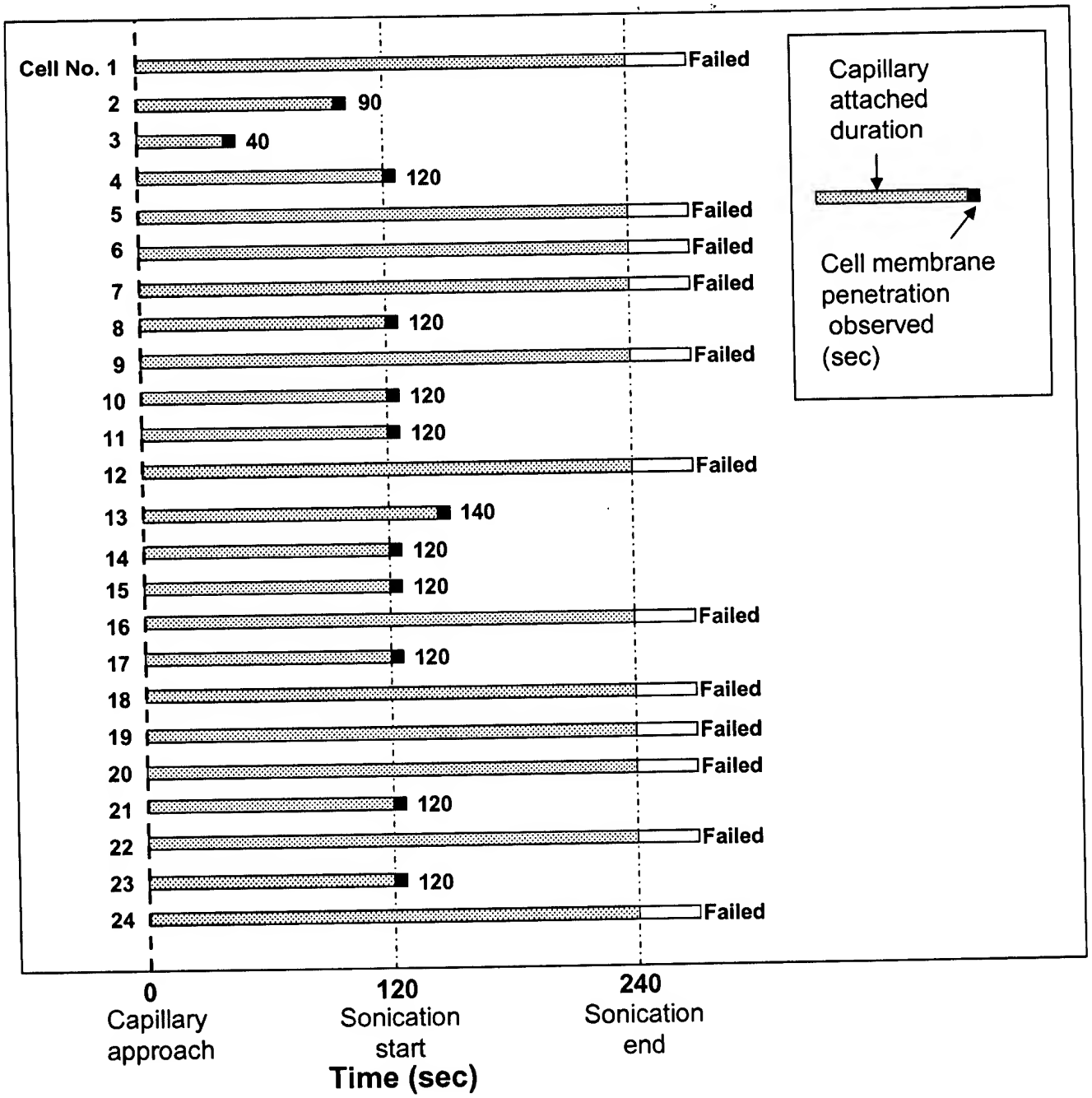

Fig. 2

Time durations until membrane-penetration observed without sonosensitizer (Control experiments)

Injection components : Marker only

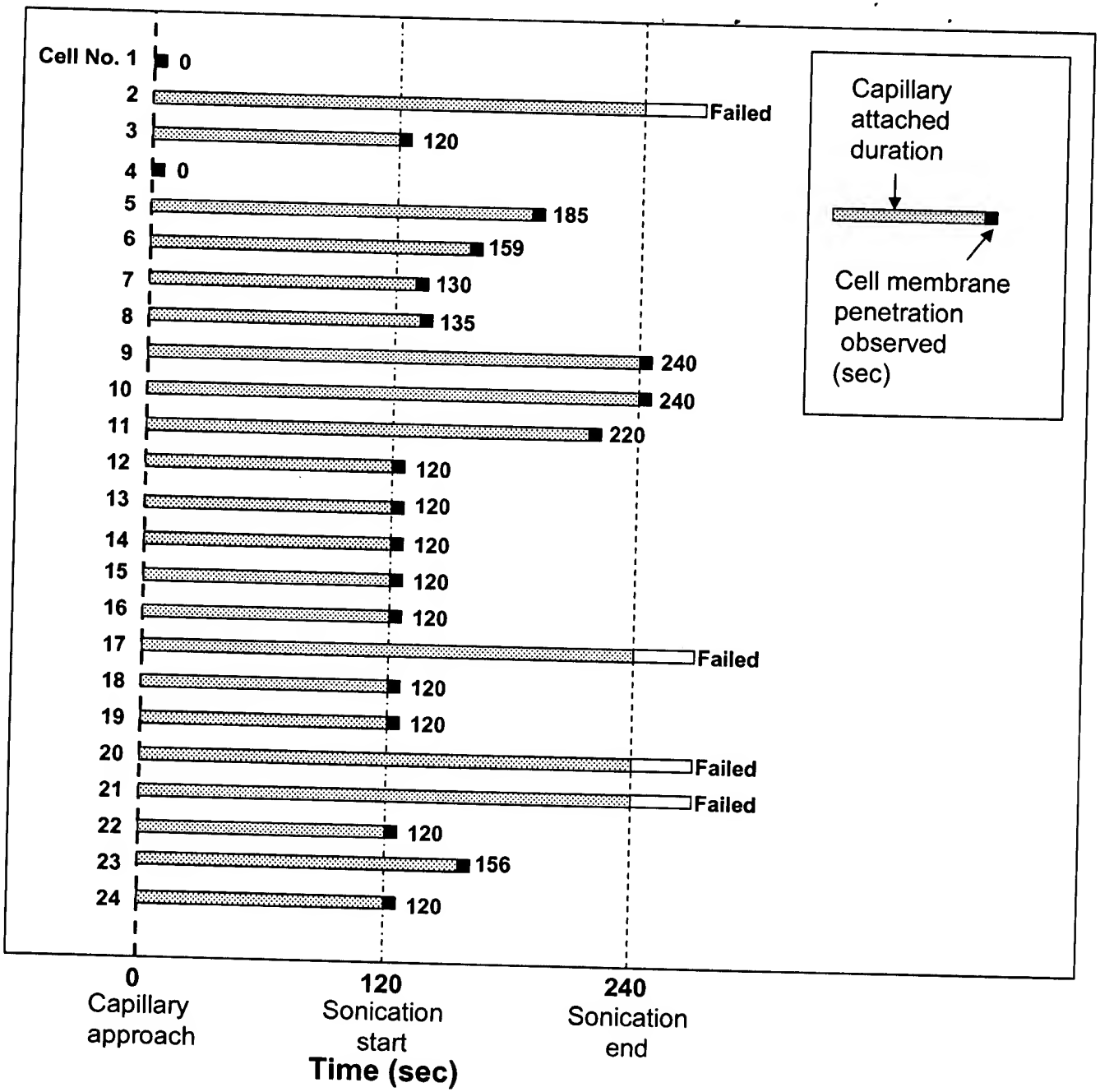

Fig. 3

Time durations until membrane-penetration observed with Sonosensitizer

Injection components :
Marker + Sonosensitizer

Table 1. The cell numbers classified by supposed membrane penetration mechanisms

Injection components	Group A $0 \leq t < 120$ *1	Group B $t = 120$	Group C $120 < t \leq 240$	Group D Injection Failed ------	Total cells
Marker + Sonosensitizer	2	10	8	4	24
Marker only (Control)	2	9	1	12	24

*1: "t" is the time when the cell membrane penetration was observed (sec).